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ARTICLE

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Aging dynamics in globular proteins: summary and analysis of experimental results and simulation by a modified trap model

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Abstract Recent results of spectral diffusion experiments by spectral hole-burning techniques carried out at cryogenic temperatures on various monomeric heme proteins unequivocally show interesting new features of conformational dynamics of globular proteins that were not emphasized in the literature until now. These new aspects of the protein dynamics are anomalous diffusion and the aging effect. Here, using the similarities between proteins and glassy systems, we present a model which can interpret the line broadening and—through this effect—the aging phenomenon as well. Leaving untouched the widely accepted energy landscape (EL) concept for the general description of protein dynamics, we concentrate on the bottom of the funnel-like EL, because this part corresponds to the native state(s) at low temperature. We suggest that the overall shape of the EL at the lowest energy range is rather smooth, but on a finer scale it consists of traps. The dynamics is defined by sequential jumps among these traps and the process is described by a Master equation, where the hopping rate only depends on the parameters of the starting state. This model was adapted to interpret the common results of spectral diffusion experiments. We tested our model in the simplest case by computer simulation, and it shows excellent agreement with the experimental data. To our knowledge this is the first work where a theoretical interpretation of the aging dynamics of proteins is directly and quantitatively related to the experimental observations. We also show that the model, after the generalization that the traps are hierarchically organized, is in accordance with the concept of other well-known EL models.

Keywords Anomalous diffusion · Energy landscape · Spectral diffusion · Spectral hole burning

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Introduction

From a scientific point of view, studying the conformational dynamics of proteins is important because their biological functions are, almost without exception, accompanied by conformational changes. Beside their specific structures, the inherent fluctuations that are indicated by experimental findings and theoretical arguments enable proteins to execute many of the tasks required for biological functioning. Traditional X-ray crystallography methods provide data for the best representation of a protein's average structure. The atoms of each protein molecule exhibit sizable fluctuations about this average that can also be estimated from the diffraction pattern (Frauenfelder et al. 1979). In some cases, computer simulations of the atomic motions in a protein have shown that the activity of the protein would be impossible if the molecule were fixed in its average structure (Karplus and McCammon 1986). The sequence of amino acids more or less determines the average structure of the protein, but the description of the dynamics of the system could happen at different levels. On an atomic level we should know all the attractive and repulsive forces among the individual atoms of which the amino acids are composed. It is clear that, because of the complexity of the system, this cannot be executed. The molecular dynamics simulations involve reasonable approximations and they are nowadays widely used approaches to protein dynamics. Another possibility for describing the dynamics of

complex systems are the phase-space models, where the dynamics of the whole system is summarized in the motion of a single point evolving within a complicated energy landscape (EL) in configuration space.

Energy landscape and conformational substates

Most often the EL is defined as the potential energy of the system as a function of all coordinates, which are needed to specify a conformation. It could be considered as a surface in multidimensional space. The high dimensionality of this representation reflects the many degrees of freedom of a protein chain. Each point on the EL represents a certain protein conformation. Conformations that are similar geometrically are close to one another on the spatial coordinates of the EL, but may be different in energy. On the other hand, very different chain geometries can result in similar energies.

Protein folding is a very interesting and currently studied process related to conformational dynamics. The main question of this topic is how can a globular protein find its final native structure from a one-dimensional chain resulting from protein synthesis, or caused by denaturation. A revolutionary "new view" of the kinetics of protein folding was the idea that the simple "folding pathways" should be replaced by funnel-like ELs (Bryngelson et al. 1995; Dill and Chan 1997). In this model, a funnel accounts for the progressive reduction in dimensionality of the accessible conformational space, beginning from the many degrees of freedom available to the unfolded chains, ultimately down to the "nearly complete lack of freedom" of the native conformation. One has to note also that, not so "far" from the funnel, there could be other, shallower local minima, meaning different intermediate (unfolded or misfolded) states (Thorn-Leeson et al. 2000).

Although in protein folding studies it is often supposed that the native structure is an unambiguously defined state, experimental results demonstrated that even the folded protein assumes a very large number of different structures or conformations called conformational substates (CSs) (Frauenfelder et al. 1979, 1988, 1991; Gafert et al. 1995a). Proteins in different CSs have the same coarse structure but differ in local, atomic configurations. Thus the EL at the bottom (EL_b) can be considered as a representation of a large number of CSs.

Describing and understanding the conformational dynamics of a protein in the native state means to determine the structure of its EL_b. Because of the complexity of such systems, a detailed geometry or topology of the EL_b cannot be expected. The only possibility is to provide a qualitative picture of EL_b, characterizing its structural parameters (for instance, the heights of barriers) that are comparable with experimental data.

Proteins are quite often compared to glasses and spin-glasses (see e.g. Frauenfelder 1986; Schlichter and Friedrich 2001). Indeed, in many respects they show similar behavior. These similarities can be used when we construct our EL_b concept for proteins. Several statistical mechanical models have been developed for describing the specific (off-equilibrium) dynamics in glassy systems, that is, the aging effect (Bouchaud et al. 1998). We think that if the EL_b is constructed on the basis of these models, it will inherit all the good features of them.

Materials and methods

Spectral hole burning as a powerful technique

Optical spectroscopy, especially the energy-selected (line narrowing) techniques, provide great potential for the study of protein dynamics. Several works are available that describe the general principles behind these techniques, as well as their particular merits for the study of structurally disordered systems (Jankowiak and Small 1987; Zollfrank et al. 1991; Thorn-Leeson and Wiersma 1994; Friedrich 1995). These techniques are based on the fact that the electronic transition energy of a marker molecule embedded in the protein is modified by the protein environment from molecule to molecule, and also in time, because of the statistically occurring conformational transformations. We can see directly this effect by conventional optical spectroscopy: instead of a characteristic sharp line, a broader spectral band appears (a summation of many individual spectral lines). This spreading of the transition energies is called inhomogeneous broadening.

Persistent spectral hole burning is an energy-selected method that requires a marker molecule with a rather long-lived intermediate in its deexitation pathway (photochemical or a structural state: product state), whose absorption spectrum is different from that of the original state (educt state) and which is optically accessible. In the experiment, laser irradiation transfers a population to this intermediate. Since this population transfer is accompanied by a frequency transformation, a depletion dip, a so-called spectral hole, will appear in the spectrum at the laser frequency after the rescanning of the same spectral range (see Fig. 1).

At sufficiently low temperatures (e.g. at a few K) the intermediate states become rather stable and the hole width may be very sharp, i.e. orders of magnitude narrower than the inhomogeneous width. Thus perturbations on this scale can be investigated by this method. Beside the high spectral resolution, the other advantage of this method is the persistency of the holes that allows for experiments with nearly unlimited time scales.

Spectral diffusion of heme proteins

In the case of heme proteins, the marker molecule can be the prosthetic group of the protein; however, a problem arises that the natural heme group (iron-heme) is not suitable for high-resolution spectroscopy. Because of the very short (fs) lifetime of the excited states, the corresponding spectral lines are immeasurably broad. To circumvent this problem, the iron-heme was substituted with Zn, Mg or metal-free porphyrins in these studies. The new chromophore serves as an ultrasensitive probe for the continuous structural rearrangements of the protein. There are many studies that clearly show that the resonance frequencies of the chromophore are strongly coupled to the conformation of the apoprotein (Friedrich et al. 1994; Gafert et al. 1995b). Even small structural fluctuations of the protein, expected at low temperature, lead to measurable fluctuations of the optical resonance frequencies of the chromophore. A selected transition frequency (burning frequency) may also be sensitive to these changes. This phenomenon is a kind of a random walk in the frequency space, which manifests itself in the broadening of the hole. It is called spectral diffusion.

When a spectral hole is created, a special group of marker molecules are selected by narrow line-width laser excitation. Through these marker molecules, a group of protein molecules are also selected, but they are not removed physically and only become

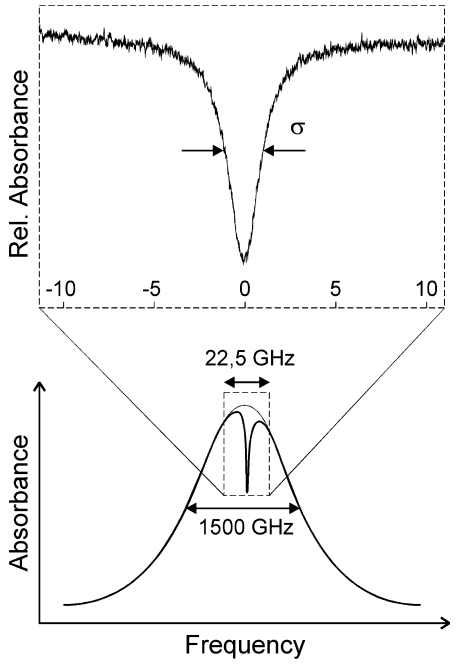

Fig. 1 Schematic representation of an inhomogeneously broadened absorption band before and after hole burning. In the *magnified frame* a real spectral hole (the difference of the two spectra measured at 4 K on mesoporphyrin-substituted horseradish peroxidase as an example) can be seen. Typical frequency ranges and the meaning of σ are also presented

invisible in the inhomogeneously broadened absorption band. Upon waiting and remeasuring, the broadening of the hole is observable. Naturally, this diffusion-like process depends on temperature. In a spectral diffusion hole-burning experiment the hole-width (σ) is measured at constant temperature as a function of the time elapsed after burning ("waiting time" t_w).

It seems reasonable that the dynamics of this time-dependent hole-broadening reflect the dynamic features of the protein in the real space. It also should reflect the specific features of the EL_b in the configuration space on which the system is wandering, i.e. its general shape, roughness, the height of the respective barriers, etc. More information can be obtained if the waiting time experiment is combined with "aging", which means that it is performed by burning holes at different "aging times", t_a, measured from the moment when the cooled sample has reached its final temperature. Thus we could study whether the response of the system to a perturbation (hole burning) also depends on the thermal history. The respective process yields an additional contribution to spectral diffusion, so the hole width may depend on both t_w and t_a (here we note that the nomenclature is not uniform in different fields of science: "waiting" and "aging" time are often mixed).

Summary and analysis of experimental results

Friedrich and co-workers performed spectral diffusion hole-burning experiments through extremely long time periods (more than three orders of magnitude on the time scale). Results on three heme proteins, myoglobin, cytochrome *c* and horseradish peroxidase, have been reported (Fritsch et al. 1996a, 1998; Thorn-Leeson et al. 1997; Schlichter et al. 1999, 2000a, 2000b, 2001, 2002). The experimental results show some small differences, but the main features are common.

Figure 2 (panels 1) shows a typical spectral diffusion waiting time experiment for a protein at low temperature in a normal (A1), in a linear-log (B1) and in a log-log plot (C1): the change of the hole-width, $\Delta\sigma$, as a function of waiting time, t_w [the data points are taken from experiments on mesoporphyrin-substituted horseradish peroxidase as an example (Schlichter et al. 2001); the three types of representation are needed for the following considerations]. From the first representation (Fig. 2-A1) it is clearly seen that there is not any indication that broadening would saturate. From Fig. 2-B1 we can see that the broadening of the hole, $\Delta\sigma$, has a quicker divergence than a $\log(t_w)$ law (otherwise it would be a straight line in this representation). The straight line on the double-log representation in Fig. 2-C1 unambiguously demonstrates that spectral diffusion broadening in the proteins is described by a power law ($\log \Delta\sigma = \alpha \log t_w + \text{const} \rightarrow \Delta\sigma \sim t_w^{\alpha}$). From the fit it was found that $\alpha \approx 0.25$–0.35, more or less independently of the type of protein [the exponent is more sensitive to the fitting concepts and procedures than to the difference in proteins; see Schlichter et al. (1999)].

Figure 2-B1 also shows two additional series of spectral diffusion waiting-time experiments combined with aging for the proteins (in the most frequently used linear-log representation). The aging times vary from some hours to roughly a week. This series of experiments shows that there is a significant aging effect, which means that spectral diffusion broadening decays as a function of aging time, t_a, for a fixed waiting time, t_w.

In the following we underline some important features of these experimental observations:

1. No saturation (see Fig. 2-A1). Proteins are spatially confined systems characterized by typical length scales of a few nm. Since their conformational phase space is also confined, the mean-square frequency displacement should saturate at some waiting time, t_w, and the spectral diffusion broadening cannot go on forever. It is easy, however, to estimate that the protein in these experiments is far from such a situation. Given that the saturation limit is the inhomogeneous width, which is typically larger than 1500 GHz, and the maximum of the measured hole width is just a few GHz, the protein is by orders of magnitudes far from this limit.
2. Power law versus logarithmic law of broadening (see Fig. 2-B1). In many situations the source of the frequency modulation which is a dominant mechanism of line broadening in condensed phases can be modeled by flipping two-level systems (TLSs) interacting with the chromophore. In the standard TLS model the configurational changes are described as tunneling transitions in an ensemble of double-well potentials whose energies and relaxation rates are widely distributed (Anderson et al. 1972). On the basis of this model, the theory predicts a logarithmic time dependence of the optical line-widths, which has been observed for several organic glasses (Fritsch et al. 1996b). The concept of the standard TLS model fails for proteins, as they are described by a power law behavior in time.
3. Exponent α (see Fig. 2-C1). There are experimental indications that protein dynamics is governed by classic-like diffusion processes even at temperatures as low as a few K (Thorn-Leeson and Wiersma 1995a). In the case of normal diffusion in real space, the variance of the position of the diffusing particle depends linearly on time ($[< x_t^2 > - < x_t >^2] \sim t$). If the broadening were governed by normal diffusion in frequency space, then, similarly to particle diffusion in real space, the mean-square displacement of the transition frequency would also evolve linearly in time. This means that the broadening would follow $\sqrt{t}$ dynamics and α would be equal to 0.5.
4. Aging (see Fig. 2-B1). The normal diffusion concept also contradicts the observed aging effect: the system being in "equilibrium" on one hand, but observing very long "relaxation" times on the other, would not exist simultaneously. In other words, the mean-square frequency displacement should not depend on the aging time t_a.

Finally, Fig. 3 could be considered as a summary of spectral diffusion experiments combined with aging for the proteins. It is represented (after the fitting procedure) by a two-dimensional surface, $\Delta\sigma$ (t_w,t_a). For comparison, we also represented the similar surface of normal diffusion to see the deviations better.

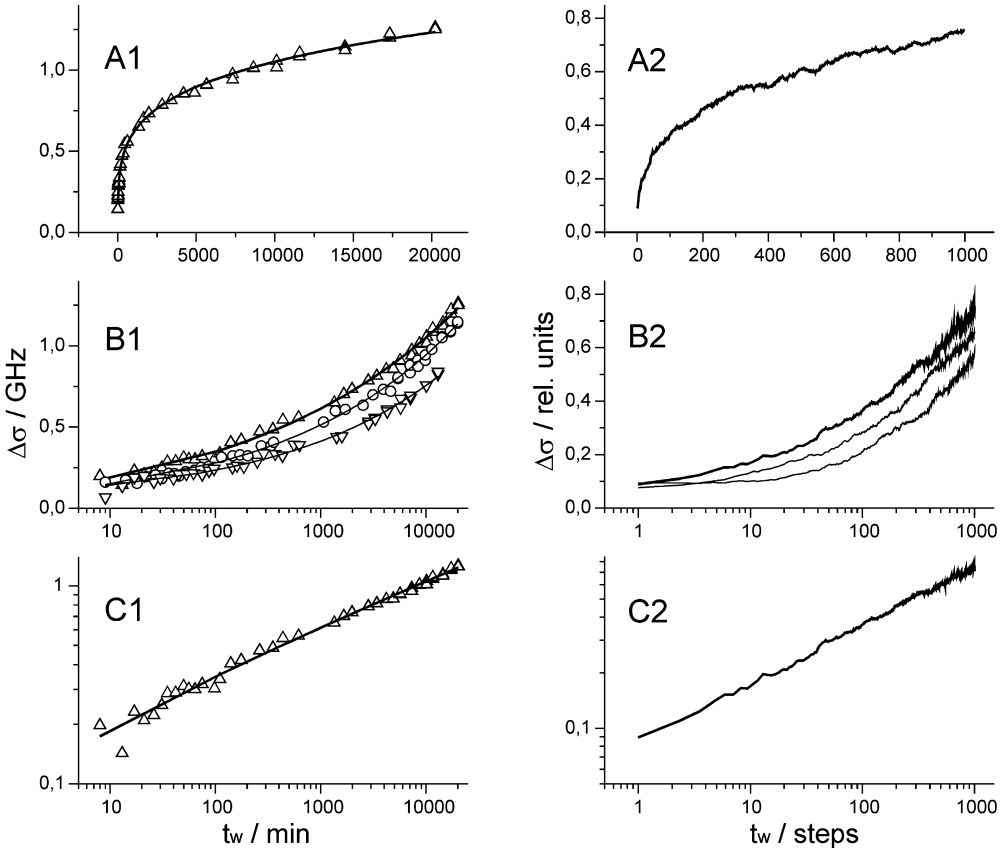

Fig. 2 *Left panels* (*panels 1*): a typical spectral diffusion waiting-time experiment for a protein in a normal (**A1**), in a linear-log (**B1**) and in a log-log plot (**C1**): the change of the hole width, $\Delta\sigma$, as a function of waiting time, t_w. The *data points* are taken from experiments on mesoporphyrin-substituted horseradish peroxidase at 4.2 K as an example. *Open symbols* mean the data measured and the solid curves are the fitted functions. In part **B1** can be seen two additional series of spectral diffusion waiting-time experiments combined with aging (these fitted functions are represented by *thinner lines*). The various aging times reach from some hours to roughly a week. *Right panels* (*panels 2*): results of simulation represented on the same way as the experimental data in the left panels, namely in a normal (**A2**), in a linear-log (**B2**) and in a log-log plot (**C2**): the change of the hole width, $\Delta\sigma$, as a function of waiting time, t_w. In part **B2** can be seen two additional series with two different aging times

The features listed above point to two important questions. (1) How could the exponent deviate from 0.5 in such a diffusion-like process? (2) How could we interpret the aging effect?

A concept for interpretation

Although the spectral diffusion happens in frequency space, it is a consequence of the conformational "motions" of the protein as a complex system. The assumption that spectral shifts of the chromophore correlate with conformational changes of the proteins has been made before (Campbell et al. 1987). A direct connection between the mean-square displacement of the change in the absorption frequency of the dye probe and the mean-square displacement of the change in the distances of the surrounding atoms could also be shown through a simplified picture of a diffusion model (Schlichter et al. 2000b).

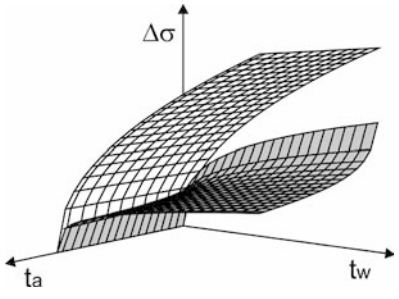

Fig. 3 A calculated (based on the experimental data) two-dimensional surface of $\Delta\sigma(t_w, t_a)$ (*gray*), and a similar one for normal diffusion (*white*). It could be considered as a summary of spectral diffusion experiments combined with aging for proteins

Keeping this in mind, we want to find the typical EL_b structure that explains the results in frequency space. Plainly speaking, the process of spectral diffusion deviates from normal diffusion; thus it belongs to anomalous diffusion. An excellent review of this topic is available that deals with Brownian motion in inhomogeneous media (Bouchaud and Georges 1990). We learn from this paper that for cases in which the inhomogeneities can be modeled as a quenched disorder on the local hopping rates, the usual form of the central limit theorem has to fail whenever anomalous diffusion occurs. This can be due to the presence of "broad distributions" (with a diverging first or second moment).

Beside anomalous diffusion, aging is also a common feature of the dynamics of complex systems like glasses and proteins, as we can see. It can be most easily understood by considering quantities depending on two time variables, e.g. correlation or response functions. In a system without aging, and after some equilibration period, these quantities depend only on the difference of the two times. In contrast, aging manifests itself in a full dependence on both time variables, resembling dynamics, which never reaches equilibrium.

In these models the free energy is given by the form: $F = Nf + f_\alpha +$ negligible terms. The first term is the extensive part of the free energy and the next is the so-called subextensive part (f_α). This is what we want to study and consider as trap-like conformational substates with a given $p(f_\alpha)$ distribution (at low temperature the extensive term does not play an important part). We could identify the dynamics in this concept as a fluctuation around the native state, namely at the bottom of the folding funnel (EL_b). We suggest following the concept of anomalous diffusion and constructing a possible model for the structure of the EL_b in this way.

Results and discussion

Trap model and its adaptation

The trap model is a highly simplified phase-space model for the dynamics of complex systems (Bouchaud 1992; Bouchaud and Dean 1995; Bouchaud et al. 1998). The energy landscape in configuration space (in a coarse-grained representation) consists of traps separated by barriers, from where the system can only escape by thermal activation. Thus there are states α, β, γ ..., between which the system wanders (see Fig. 4A). The dynamics of the system is described by a Master equation for the probability p_α of finding the system in state α:

$$\partial p_\alpha \partial t = -\sum_\beta w_{\alpha\to\beta} p_\alpha + \sum_\beta w_{\beta\to\alpha} p_\beta \qquad (1)$$

The choice of the hopping rates $w_{\alpha\to\beta}$ then encodes the statistics of the barrier heights and the geometry of the phase-space.

Let us consider one of the simplest cases, when the hopping rate only depends on the starting state: $w_{\alpha\to\beta}=(N\tau_\alpha)^{-1}$, where N is the total number of states and τ_α is the trapping time (characteristic time of being trapped). $\tau_\alpha = t_0\exp(B_\alpha/kT)$, where t_0 is some microscopic time, B_α is the height of the energy barrier that surrounds state α (or the depth of the trap), k is the Boltzmann constant and T is the absolute temperature. The final state β is thus independent of the initial state (it can be chosen randomly), and the process starts anew at each jump. Within this description, the equilibrium measure $p^{\mathrm{eq}}{}_\alpha$ is simply proportional to τ_α. In order to reproduce the correct Boltzmann equilibrium, one should thus identify B_α with $|f_\alpha|$. Several reasons suggest that the $p(f_\alpha)$ distribution is exponential: $p(f_\alpha)\sim\exp(-x|f_\alpha|/kT)$, where the parameter x is a monotonic increasing function of temperature [it is a general property of glassy systems (Derrida 1981)]. It can be shown that at low temperature (in a glassy phase) for $x<1$ the system "ages", which is reflected by the fact that the distribution of the trapping times becomes so broad (the average trapping time becomes infinite) that the sum of all τ_α for the trapping events, $\tau_1+\tau_2+...+\tau_N$, is always dominated by its largest term.

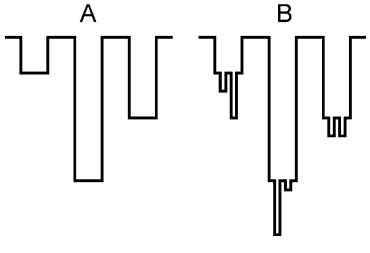

Fig. 4 Schematic phase-space landscape (EL_b) for the trap model: **A** the simplest case ("one level tree"); **B** traps are organized on a hierarchical tree ("multi-level tree")

This model was adapted in a modified version for our purposes under a special condition. Within an inhomogeneously broadened spectral band, each frequency of the spectrum (ν), and thus each transition energy of the marker ($E=h\nu$), represents more than one protein conformation; therefore, these "spectral states" are degenerate. As was mentioned above, the protein conformations that are similar geometrically are close to one another on the spatial coordinates of the EL_b and in the transition energy of the marker (E) also (see Schlichter et al. 2000b), but may be different in potential energy. Let us identify the α, β, γ, ... states in the trap model with the CSs of the EL_b and the energy barriers of these states (B_α, B_β, B_γ, ...) with the potential energy of the CSs of the EL_b. Then we apply the simplest trap model in a little bit modified but more realistic form: after the trapping time, just those states will be chosen, randomly, which have a rather similar E parameter (close "spectral states").

Summarizing our model:

1. EL_b consists of states (α, β, γ, ...).
2. Each state is characterized by two parameters $\{E_\alpha|B_\alpha\}$.
3. The hopping rate only depends on the starting state α and inversely proportional to the trapping time: $w_{\alpha\to\beta}\sim(\tau_\alpha)^{-1}$.
4. The average trapping time is determined by the formula: $\tau_\alpha\sim\exp(B_\alpha/kT)$, where B_α is the depth of the trap.
5. The final state β can be chosen randomly with a condition that E_β may not be far from E_α.
6. The process starts anew at each jump.

Note that the original trap model has no real geometry. In our version the geometry is defined by the condition in 5: close protein conformations means closeness of the E parameters (close frequencies in the spectrum). At this point, we stress the important characteristics of the dynamics: while the parameter E changes quasi continuously, as is expected for a *local* quantity, the (subextensive) parameter B has finite jumps, as a direct consequence of its *collective* nature.

Here we have to mention a model for the relaxation dynamics of myoglobin following photodissociation of bound CO, where the authors use very similar basic assumptions (Hagen and Eaton 1996).

Simulation and its comparison with experimental data

All the states $\{E_\alpha|B_\alpha\}$ of an ensemble which is studied by a spectral diffusion experiment can be arranged on a two-dimensional scheme, where E_j increases from left to right in each row, but it is constant in a column, and B_i is optional. Summing up the p_α probabilities along a column ($\sum_{E_j=\mathrm{const}} p_\alpha = p_j$), the relative population of a "spectral state" will appear and the ranked set of these sums (according to E_j), $p_j(E_j)$, represents the absorption spectrum.

Suppose that, in the initial state of the system, all p_α are equal. Uniform distribution is a usual choice for initial states in quenched disordered systems, which corresponds to Boltzmann distribution, at high temperature ($T=\infty$). Naturally it will not be an equilibrium distribution at low temperature (because of the rather quick cooling procedure in the spectral diffusion experiments, it is a realistic assumption for our protein systems). From the point of view of the simulation, it means that all the states $\{E_\alpha|B_\alpha\}$ for the ensemble are equally populated.

The dynamics is defined by the following steps:

I. Three random numbers (RN) were generated for each state in the [0,1] interval.
II. If $RN_1 \leq w_{\alpha\to\beta}$ the system can escape from state α (if $RN_1 > w_{\alpha\to\beta}$ the state remains unchanged).
III. RN_2 determines the new position along the columns (vertically) on the scheme.
IV. RN_3 determines the new position along the rows (horizontally) on the scheme.
V. After that, the jumps happen independently from one another but simultaneously for all the states (time step).

Note for step II: the hopping rate ($w_{\alpha\to\beta}$) was calculated by points of model 3 and 4. We tried and applied different distributions for B, but the uniform distribution gave the best results (those are represented in Fig. 5). The uniform distribution corresponds to $x=0$ for $p(f_\alpha)$ in accordance with the fact that the experiments were performed at very low temperature (anyway, it results in the broadest distribution of trapping times).

Note for step IV: according to the special condition (points of model 5), we also applied different methods, but for most of the cases horizontally, just the nearest neighbor states were chosen with equal probability. Thus we checked if RN_3 is smaller or larger than 0.5.

A spectral hole-burning event at a certain aging time (t_a) can be simulated by decreasing the population near a certain E_j parameter in each row (for simplicity we applied Lorentzian hole-shape at the middle of the "spectral" range). Figure 5 shows four simulated spectra ($p_j(E_j)$) at different times and two holes with the fitted curves. After each time step the simulated hole was fitted by a Lorentzian and the difference between the determined actual and initial hole widths (the change of the hole width, $\Delta\sigma$) was represented as a function of waiting time (t_w).

In Fig. 2 (panels 2) we can see the results of the simulation for the broadening process. To make the comparison easier, they are represented in the same way as the experimental data in Fig. 2 (panels 1). The similarity is conspicuous at first sight. After quantitative analysis of the simulation data, we obtained the exponent of the power law dynamics: $\alpha\approx 0.3$. It even shows a good quantitative agreement with the fitted α parameters of the spectral diffusion experiments.

We can see that the model works, which means it can describe the dynamic features of the system properly,

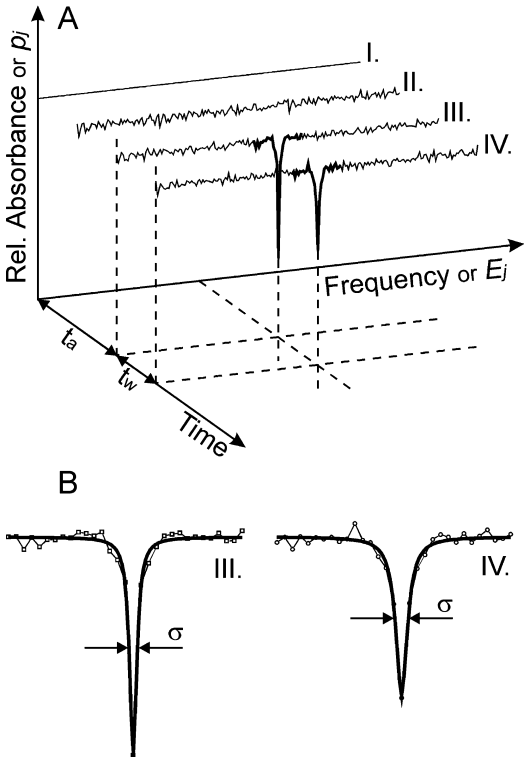

Fig. 5 **A** A series of simulated spectra at different times: I, initial; II, intermediate before hole burning; III, just after the hole-burning event (initial hole); IV, after a certain waiting time (broadened hole). **B** Determination of hole width: the middle parts of the spectra in **A** III and IV (*thicker lines*) are magnified and fitted by Lorentzians

namely the anomalous diffusion and aging. Thus the model answers the two main questions in the previous section.

Comparison with other EL concepts

Frauenfelder and co-workers suggested first that the EL of proteins is hierarchically organized (Ansari et al. 1985). They consequently expect the EL to be arranged in a hierarchy of tiers, with different tiers having considerably different barrier heights between substates. According to this concept, every CS within a particular tier in the hierarchy is split into a number of substates which comprise the next tier of the CSs. The average height of the energy barriers between CSs within one tier decreases for descending tiers.

Several kinds of experiments have provided additional data about the barriers from higher to lower levels of the EL, especially temperature-derivative spectroscopy (Berendzen and Braunstein 1990; Herenyi et al. 1995), three-pulse stimulated photon echoes (Thorn-Leeson and Wiersma 1995a) and temperature-cycling hole-burning experiments (Kohler et al. 1998). Theoretical results also supported this hierarchical concept (Berlin and Burin 1997; Becker and Karplus

1997; Wales et al. 1998; Frauenfelder and Thorn-Leeson 1998; Miller and Wales 1999; Frauenfelder and McMahon 2000).

Nevertheless, there are research results which seem to contradict the hierarchy. For example, the configuration coordinate model assumes the harmonic EL can give a fairly good description of the equilibrium conformational fluctuation of Zn-substituted myoglobin studied by time-resolved transient hole-burning spectroscopy (Sibata et al. 1999). It was found that the activation energy for the fluctuation process is almost zero in the 180–300 K temperature region. These results are not so surprising and they are understandable if we take into consideration that the experiments were done at relatively high temperatures. In this temperature range the fine structure of the EL is unobservable, and thus cannot be tested. Consequently, no contradiction arises.

Thorn-Leeson and Wiersma (1995b), however, propounded that the energy landscape may be more strictly ordered than is implied by a hierarchical organization of the CSs. Based on their experimental results, these authors suggested that the potential energy surface is self-similar in terms of the relation between barrier heights that characterize the successive tiers. Although Frauenfelder (1995) raised doubts about this suggestion, we think that the spectral diffusion experiments of Friedrich and co-workers perhaps may provide evidence for the self-similar structure of the EL_b. Namely, some of these experiments were performed at two different temperatures and although the spectral diffusion width at 4 K is by more than an order of magnitude larger than at 100 mK, it follows the same power law in time with the same exponent (≈ 0.3) (Schlichter et al. 1999). Here we have to emphasize that the temperature (and also the effective time scale) at which the experiments are conducted is a crucial factor. It would be a good idea to perform these spectral diffusion experiments over a wider temperature range, but because of the "extremely long time periods" (about two weeks, see Fig. 2-A1 and in the "Summary and analysis of experimental results" section), it is almost insolvable. Note that keeping all the parameters constant for such a long time is enormously hard work.

Although we applied the simplest version of the trap model ("one-level tree" model) for the simulation, we can show that by a generalization of the model ("multi-level tree" model) it is in accordance with the concept of the hierarchical EL models.

Consider a hierarchical organization of traps within traps (see Fig. 4B). The hopping rate $w_{\alpha \to \beta}$ is still taken to be independent of the final state for a given distance along the tree. If j labels the level of the tree from top to bottom, then a certain j^* divides the hierarchy into two parts: all the levels $j > j^*$ are equilibrated on microscopic times ($\sim t_0$) (this part of the tree thus contributes to the stationary dynamics), while the levels $j < j^*$ contribute to the aging part. This concept fits well with the overall hierarchical EL models.

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